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Expression of Nogo-A Is Decreased with Increasing Gestational Age in the Human Fetal Brain

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Key Words
Nogo-A protein · Axonal regrowth inhibitor · Human fetal brain · Reticulon family

Abstract
Nogo is a member of the reticulon family. Our understanding of the physiological functions of the Nogo-A protein has grown over the last few years, and this molecule is now recognized as one of the most important axonal regrowth inhibitors present in central nervous system (CNS) myelin. Nogo-A plays other important roles in nervous system development, epilepsy, vascular physiology, muscle pathology, stroke, inflammation, and CNS tumors. Since the exact role of Nogo-A protein in human brain development is still poorly understood, we studied its cellular and regional distribution by immunohistochemistry in the frontal lobe of 30 human fetal brains. Nogo-A was expressed in the following cortical zones: ependyma, ventricular zone, subventricular zone, intermediate zone, subplate, cortical plate, and marginal zone. The number of positive cells decreased significantly with increasing gestational age in the subplate and marginal zone. Using different antibodies, changes in isoform expression and dimerization states could be shown between various cortical zones. The results demonstrate a significant change in the expression of Nogo-A during the development of the human brain. The effects of its time- and region-specific regulation have to be further studied in detail.

Introduction

Reticulons are a diverse family of proteins, all containing a highly conserved reticulon homology domain at the carboxy terminus but with highly variable N-terminal sequences (reviewed by Yang and Strittmatter [1]). As a member of this protein family, Nogo contains the conserved domain but it is the unique behavior of the nonconserved domains that are of most interest to this study. Since the discovery of Nogo over a decade ago, it has become clear that it serves a prominent role in neurodevelopment as a regrowth inhibitor [2]. This function has outcomes relevant to a wide range of disorders, including epilepsy, vascular physiology, muscle pathology, stroke, inflammation and central nervous system (CNS) tumors.

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Three isoforms of Nogo (Nogo-A, -B and -C) exist that arise from a single gene via alternative splicing or alternative promoter usage. All of them are members of the reticulon family [3]. Nogo-A is known to be expressed by oligodendrocytes and neurons and is present on oligodendrocytes in the inner and outer loops of the myelin sheath [3, 4]. Nogo-A has two transmembrane components with an intervening 66-amino-acid domain. The latter domain is thought to be extracellular, but its exact topology has not yet been clarified [5]. Two domains have neurite growth-inhibitory properties, the 66-amino-acid extracellular loop (Nogo-66) and its N-terminal region (Amino-Nogo). Amino-Nogo requires immobilization to a substrate and dimerization for it to be effective as a neurite outgrowth inhibitor, but this is not the case for Nogo-66, being able to induce growth cone collapse in soluble form [6]. Although the extracellular location of Nogo-66 is deemed to enable the inhibitory effects of Nogo-A, CNS injury inevitably leads to myelin destruction and exposure of Amino-Nogo as well. The Nogo receptor (NgR) mediates the inhibitory action of Nogo-66 [6]. The NgR is a glycosylphosphatidylinositol-anchored protein that associates with p75 neurotrophin receptor [7]. In addition to inhibition of neurite outgrowth, these molecules have other functions. Nogo-A, MAG and OMgp are localized at distinct axonal domains and are involved in axoglial interactions [8, 9].

In the adult human nervous system, Nogo-A is expressed predominantly in oligodendrocyte cell bodies and myelin sheaths, and to some extent in neurons of the brain and spinal cord, especially in most brain stem nuclei, dorsal root ganglion sensory cells, and spinal cord motor neurons and interneurons [10]. The presence of Nogo-A in adult neurons suggests that this protein has other roles beyond axonal growth inhibition, even in the mature CNS. These roles could include attractive or repulsive signaling for other neurons, signal transduction for unknown ligands, or some other intracellular functions [11].

During brain development, Nogo-A is known to be expressed by several neuronal populations and to have a role as a growth promoter and a fiber tract forming factor [12–15]. During early stages of myelination, Nogo appears to have a major impact on the local distribution of potassium channels in the paranodal region, through an interaction with the Caspr-F3 axoglial complex mediated by the Nogo-66 region [9, 16]. The Caspr-F3 complex is responsible for the architecture of the axolemmal-glial apparatus. The Nogo-A-Caspr complex directly interacts with $K_v1.1$ and $K_v1.2$ potassium channels and thereby influences their segregation to the juxtaparanodal region. Consistent with the view that Nogo is not involved in axonal growth at this stage of myelination, Nogo-A, but not NgR, localizes to the paranodes, and Nogo-A, Caspr and $K_v1.1$ channels have a similar spatial and temporal relationship during development. Nogo-A expression by murine radial glia and postmitotic neurons was recently described [17]. Nogo-A was not restricted to a specific radial glial population in the developing telencephalon, and both radial glia of the dorsal and ventral telencephalon expressed the protein [17]. In the study of Mingorance-Le Meur et al. [17], Nogo-A was enriched at the leading process of tangentially migrating interneurons but not in radial migrating neurons. At embryonic day (E) 12.5, Nogo-A was detected in radially oriented processes throughout the cortical lineage. At low levels, Nogo-A was demonstrated to appear on the surface of many cortical neurons. In *Nogo*-deficient background, neurons displayed early polarization and increased branching in vitro, probably reflecting a cell-intrinsic role of Nogo proteins in branching reduction [17]. Early tangential migration was demonstrated to be delayed in the same investigation. The aim of the present study was to examine the expression of Nogo-A during normal human brain development using antibodies directed against an epitope within the N-terminal region of Amino-Nogo required for dimerization [antibody 1 (Ab-1)] and an epitope adjacent to the Nogo-66 region [antibody 2 (Ab-2)]. Significant decreases of Nogo-A expression were observed with advanced gestational age (GA).

Materials and Methods

Materials

In the present study, tissue from the frontal lobe of 30 human fetal brains of various GAs was studied. The demographics of each individual as well as clinical data and neuropathological changes are listed in table 1. After the death of the patient, the brain was removed within 24 h and fixed in a 4% formaldehyde solution for 1 week. The GA of the fetuses was assessed using the gyrification pattern of the brain and compared to clinical information. Then, the brain was cut into a series of coronal sections, each of which was paraffin embedded. Routine neuropathological examination was carried out on sections stained for HE, cresyl violet and Luxol fast blue.

Antibody Generation

Polyclonal antibodies were generated to two regions of Nogo-A. The program Protean (DNaStar) was used to select optimal peptide epitopes of 15–20 residues in size. Figure 1 shows the location of the two epitopes for antibody generation (Ab-1 and Ab-2), the surface probability plot (to optimize the likelihood of epitope being available for the antibody), the coiled-coil regions (ter-

Table 1. Demographic data and neuropathological changes of the examined fetal brains

Case No.	Clin-GA	NP-GA	Gender	Clinical data	Neuropathological changes
1	18	16–19	x	Amniotic membrane infection syndrome Severe immaturity	No neuropathologic changes
2	18	16–19	f	Spontaneous birth at 18th gestational week Hyperemesis in early gestational period	No neuropathologic changes
3	18	16–19	x	No information available	Discrete subarachnoid hemorrhage Cortical verruca formations Persistence of germinal matrix cells in the frontal lobe
4	18	16–19	x	Abortus incipiens Late abortion 18th gestational week	Brain edema Cortical verruca formations
5	20	16–19	x	No information available	Cortical verruca formations
6	20	17–20	x	Imminent abortion Premature abruption of placenta	Cortical verruca formations
7	17	16–19	x	Cytogenetically proven trisomy 21	Hydrocephalus Accessory lateral ventricle (frontal) Ectopic aggregation of germinal matrical cells in intermediate areas (occipital lobe) Cortical verruca formations
8	21	20–23	x	Trisomy 21 Late abortion in 21st gestational week	Fresh meningeal hemorrhages Cortical verruca formations
9	26	20–23	f	Placental infarction Premature abruption Insertio velamentosa of the umbilical cord	No neuropathologic changes
10	22	20–23	x	Infection with toxoplasma Oligohydramnios	No neuropathologic changes
11	21	20–23	f	Cytogenetically proven trisomy 21	Germinal matrix hemorrhage Discrete vernal hemorrhages Small heterotopia of undifferentiated migrating cells
12	24	20–23	f	Premature abruption of placenta Generalized immaturity	Moderate brain edema Cortical verruca formations Persistent matrix cells Hypoxia
13	n.a.	20–23	m	Trisomy 21 Complete AV channel defect Right heart failure with pulmonary hypertension	No neuropathologic changes
14	n.a.	23–24	f	Preterm birth 23rd gestational week Intracranial hemorrhage Hyaline membrane disease Right heart failure Hydramnion	Germinal matrix hemorrhage (right side) with tamponade of the lateral ventricle, the cerebral aqueduct, the IVth ventricle Discrete circumscribed vernal hemorrhages
15	n.a.	22	f	Amniotic membrane infection syndrome Severe immaturity	Cortical verruca formations Moderate brain edema Persistence of matrical cells (temporal lobe)
16	25	24–27	m	Intrauterine hypoxia Deep placental insertion	Mild subarachnoid hemorrhage
17	24	24–27	x	Spontaneous abortion 24th gestational week	Cortical verruca formations Brain edema Persistence of matrical cells Hypoxia

Table 1 (continued)

Case No.	Clin-GA	NP-GA	Gender	Clinical data	Neuropathological changes
18	n.a.	24–27	m	Extreme immaturity Bronchopulmonary dysplasia Amniotic membrane infection syndrome	Moderate to severe brain edema Cortical verruca formations Persistent matrical cells Hypoxia Ventricular hemorrhage Germinal matrix hemorrhage
19	27		m	Lips-pin-palate column Clubfoot	Meningoencephalocele Cortical verruca formations Band (laminar) heterotopia
20	28	25–30	f	Hyaline membrane disease Intracranial hemorrhage	Hemorrhage
21	29	28–31	f	Tumor in left upper arm Hypovolemic shock	Congestion of vessels Subarachnoid hemorrhages
22	29		f	Infection Placental insufficiency	Brain edema
23	n.a.	30	m	Hypoplastic left heart insufficiency Immaturity	No neuropathologic changes
24	n.a.	32	m	Trisomy 13 Lips-pin-palate column	No neuropathologic changes
25	32	32–35	f	Intrauterine death Placental insufficiency	No neuropathologic changes
26	n.a.	32–35	m	Potter sequence	Severe brain edema Small pontine vascular malformation Residual matrical cells in the basal ganglia
27	n.a.	32–35	m	Potter sequence	Moderate brain edema Disturbed cortical architecture (temporal lobe)
28	n.a.	32–35	x	Fetal hydrops Gender uncertain	Right parietal intracerebral small circumscribed hemorrhage Severe brain edema Choroidal plexus cyst Migrational defect (frontal)
29	n.a.	36<	f	Intrauterine death	Congestion of leptomeningeal and intraparenchymal vessels
30	39	36<	m	Intrauterine death	Migrational disturbance Brain edema

Clin-GA = Gestational age as provided by clinicians; NP-GA = gestational age based on gyrification pattern; x = gender uncertain.

tiary structures that, if altered, may affect antibody binding), and the phosphorylation sites [the different colors are serine (blue), threonine (green) and tyrosine (red), with the solid horizontal line indicating threshold significance] (fig. 1). This plot was generated from NetPhos and the coiled-coil plot from COILS, both available on the net.

Nogo-A Ab-1 was an antibody generated by injecting rabbits with a peptide composed of a sequence found near the N-terminus of Nogo-A: EEEEDEDEDLEELEVLERK with a C residue added at the carboxy terminus for adjuvant purposes. The region selected was without phosphorylation and had a high surface probability.

Nogo-A Ab-2 was an antibody generated by injecting rabbits with a peptide composed of a sequence found in a more central region of Nogo-A: KVLVKEAEKKLPSDTEKE with a C residue added at the carboxy terminus to increase antigenicity. The polyclonal antibody generation and ELISA measurements were carried out by GeneMed Synthesis (San Francisco, Calif., USA). The ELISA results showed significant peptide-specific reactions at sera dilutions of 1:1 K and 1:10 K. The region was chosen because it contained minimal phosphorylation (hard to find in this protein) and lacked coiled-coil regions (see B in fig. 1). There is one likely phosphorylation site in Ab-2.

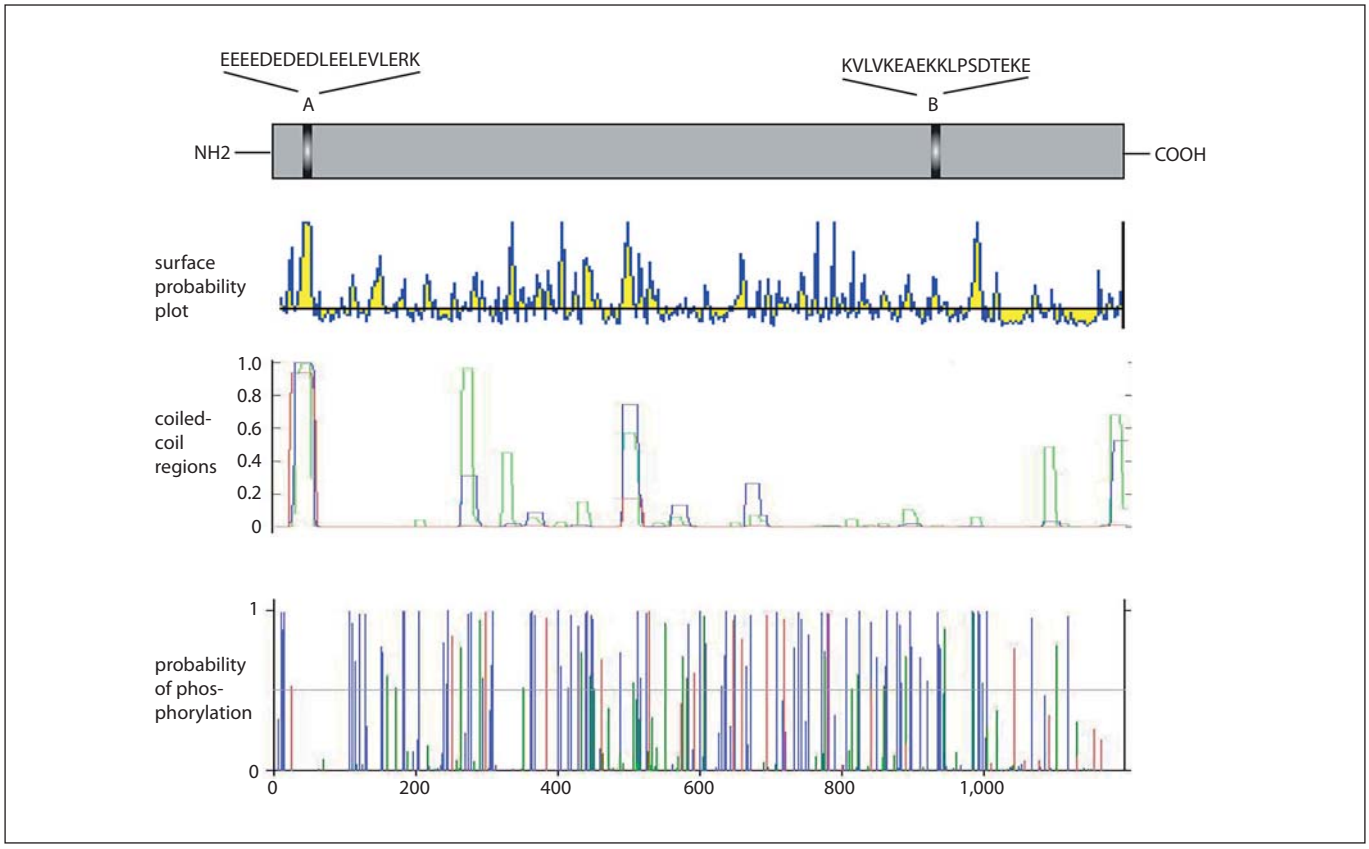

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Fig. 1. The location of the two epitopes for antibody generation (A and B), the surface probability plot (to optimize the likelihood of epitope being available for the antibody), the coiled-coil regions (tertiary structures that, if altered, may affect antibody binding), and the phosphorylation sites [the different colors are serine (blue), threonine (green) and tyrosine (red), with the solid horizontal line indicating threshold significance]. This plot was generated from NetPhos and the coiled-coil plot from COILS, both available on the net. The location of the Amino-Nogo and Nogo-66 regions are shown on the surface probability plot. For colors, see online version.

Validation Antibodies

Rabbit anti-Nogo-A ('Laura') antibody was used at 1:1,000. Sections were incubated with the secondary anti-rabbit horseradish peroxidase antibody and for visualization of the reaction product DAB was used [18].

Antibody Validation by Western Blot Analysis

Nogo-A Ab-1 and Nogo-A Ab-2 were tested by Western blotting using fresh-frozen human brain tissue in comparison to the well-established 'Laura' antibody (fig. 2) [18]. Cortex, i.e. gray matter, was compared to white matter. The deep-frozen brain tissues (gray and white matter) were lysed in NP-40 lysis buffer. The extracts were centrifuged at 10,000 rpm for 10 min at 4°C. Protein concentration of each supernatant was analyzed by Bradford assay. The protein lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and analyzed by immunoblotting using gel electrophoresis (10% gels), transferred to polyvinylidene difluoride membrane and analyzed by immunoblotting using standard methods. Membranes were incubated with the following antibodies: anti-Nogo-A Ab-1 (1:300), anti-Nogo-A Ab-2 (1:250), rabbit anti-Nogo-A ('Laura') Ab (1:20,000; self-made and kindly provided by Prof. Dr. M.E. Schwab) and anti-GAPDH (1:5,000; cell signaling). As secondary antibody, anti-rabbit-horseradish peroxidase (1:5,000; Amersham) was used.

Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed and paraffin-embedded 5-μm-thick sections on Superfrost Plus slides (M6146-Plus, Allegiance, McGraw Park, Ill., USA). Deparaffinized, rehydrated sections underwent antigen retrieval using the DAKO target retrieval solution (DakoCytomation, Carpinteria, Calif., USA, No. S1700; equivalent to a 10 mmol/l citrate buffer, pH 6.0) for 20 min in a water bath at 95–100°C. All subsequent steps were carried out using the DAKO Autostainer Immunostaining System (DAKO S3400) and the EnVison™ kit (code K4011, DakoCytomation). Sections were treated with 3% H_2O_2 for 5 min to block endogenous peroxidase followed by protein block (25% casein in PBS containing carrier protein and NaN_2, DAKO code X0909) for 5 min. The primary antibodies were used at con-

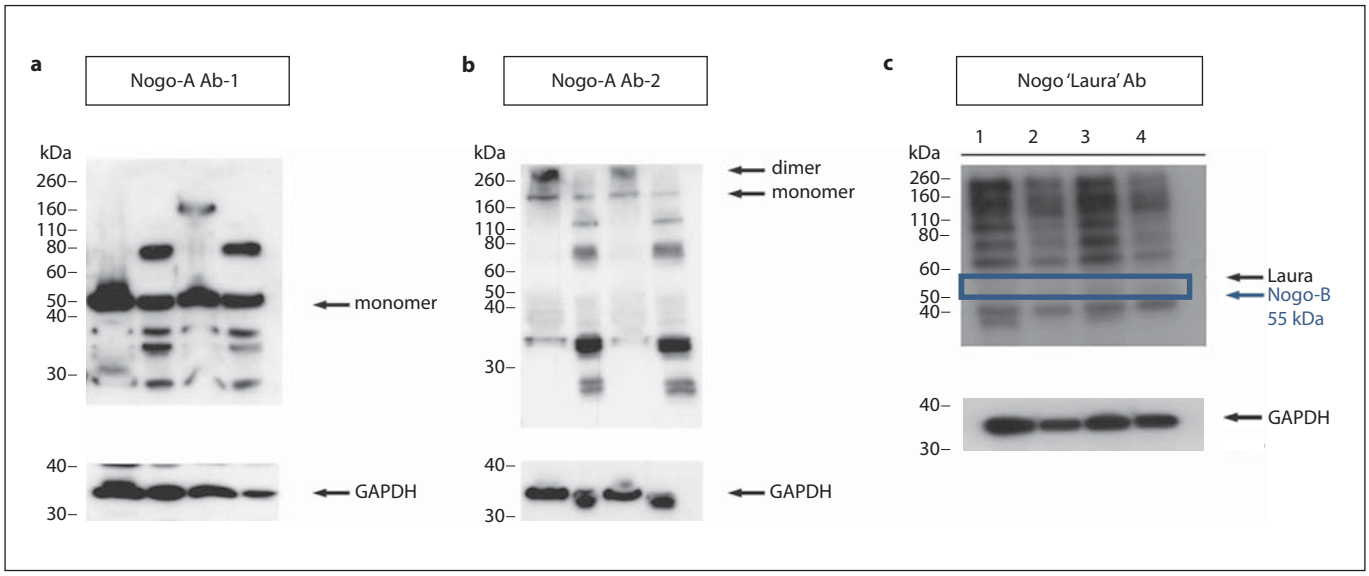

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Fig. 2. Western blot analysis of human cortex compared to white matter. **a** Nogo-A Ab-1 (1:300). **b** Nogo-A Ab-2 (1:250). **c** Nogo-A ('Laura') Ab (1:20,000). 1 and 3 represent white matter, 2 and 4 cortex (30 μg protein/lane loaded). Nogo-A Ab-2 shows enhanced dimer expression at approximately 260 kDa, while Nogo-A Ab-1 and Nogo-A ('Laura') Ab do not.

centrations of 1:300 for Nogo-A Ab-1 and 1:250 for Nogo-A Ab-2 for 30 min and 2 h, respectively. Sections were incubated with the secondary anti-rabbit antibody (conjugated with horseradish peroxidase enzyme-labeled polymer) for 30 min. The reaction product was visualized using 3,3′-diaminobenzidine chromogen (liquid DAB+, K3468, DakoCytomation) for 5 min. Then, the sections were counterstained with Gill 2 hematoxylin (Richard-Allan Scientific, Kalamazoo, Mich., USA). As negative control, the primary antibody was omitted and replaced with normal rabbit serum (code X0903, DakoCytomation).

Evaluation of the Immunohistochemical Stains

On each immunohistochemically stained section, immunopositive cells were analyzed separately for each of the following topographical locations: ependyma, ventricular zone, subventricular zone, intermediate zone, subplate, cortical plate, and marginal zone.

The staining intensity was rated as follows: 0 = no staining, 1 = weak staining, 2 = moderate staining and 3 = strong staining.

Statistical Analyses

The GAs were grouped as follows: GA1 = 16–19 weeks, GA2 = 20–23 weeks, GA3 = 24–27 weeks, GA4 = 28–31 weeks, GA5 = 32–35 weeks and GA6 = 36–40 weeks.

The differences between the GA groups were assessed using ANOVA as well as the nonparametric Kruskal-Wallis test (Statistical Package for the Social Sciences, SPSS). Post hoc testing between the various GA groups was performed using Student's t tests as well as nonparametric Mann-Whitney U test. Correlations were performed using the Spearman rank test.

Results

Both antibodies Ab-1 and Ab-2 were evaluated for their ability to detect Nogo-A in human brain tissue. Antibody Ab-1 clearly recognized a band at approximately 50 kDa (fig. 2), corresponding to the molecular weight of an isoform of Nogo-A known as Nogo-B [19]. Nogo-A (GenBank: CAB99248.1) and Nogo-B (NP_722550) share N-terminal sequences, and thus most antibodies for Nogo-A that are directed towards the N-terminal region also recognize Nogo-B.

In contrast, antibody Ab-2 is directed towards a central region that is absent in Nogo-B, and thus Ab-2 is specific for Nogo-A (no band corresponding to Nogo-B is seen in the Ab-2 results in fig. 2). This was also confirmed by the Laura antibody (fig. 2c). A BLAST search of the epitope recognized by Ab-2 reveals little else in the human proteome that Ab-2 would likely react with. The monomer for Nogo-A is 130 kDa but is known to migrate at 180 kDa [20], and can be seen in lanes 1 and 3 for Ab-2 (fig. 2). Based on the Western blot results, it seems that antibody Ab-2 is recognizing a Nogo-A dimer as the predicted molecular weight of the dimer is 260 kDa. Ab-2 is directed towards a region free of coiled-coil interactions that lead to dimer formation, whereas Ab-1 likely does not recognize this dimer because it is directed towards an epitope within the

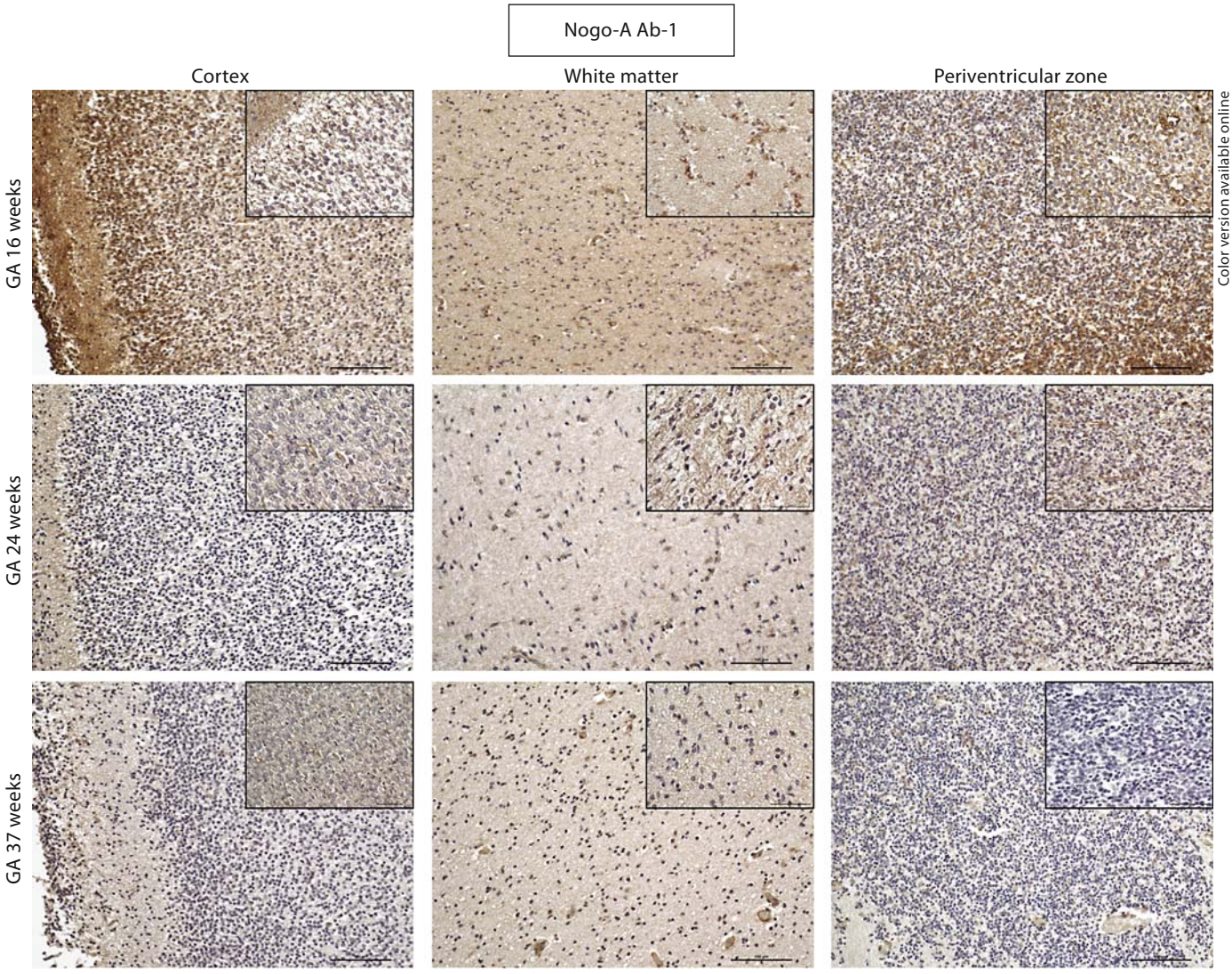

Fig. 3. Representative micrographs of Nogo-A immunopositive cells (stained with Ab-1) of three different age categories (GA 16, 24, 37 weeks) in the cortex, white matter and periventricular zone (magnifications indicated by scale bars, bar length corresponds to 100 μm; insets with higher magnification: ×40). For colors, see online version.

coiled-coil region in the N-terminus (fig. 1). When the two coiled-coil regions of the respective monomers interact to form the dimer, the epitope normally recognized by the antibody would be altered and obscured from Ab-1, whereas the epitope recognized by Ab-2 is still exposed.

Thus, the top two bands recognized by antibody Ab-2 are very likely the dimer and the monomer and the additional bands observed most probably represent breakdown products [19]. It is important to note that the dimer predominates in white matter (lanes 1 and 3 of fig. 2) but is not detected in gray matter (lanes 2 and 4 of fig. 2).

Using both antibodies, small cells with round nuclei corresponding to glial and neuronal cell types could reliably be stained (fig. 3, 4). Stained cells were located in the ependyma, ventricular zone, subventricular zone, intermediate zone, subplate, cortical plate, and marginal zone.

There was a significant difference between the two antibodies: the staining intensity was significantly higher with Ab-1 compared to Ab-2 (table 2). Based on the differences in staining, the subsequent evaluation was carried out by analyzing the results obtained with both antibodies separately.

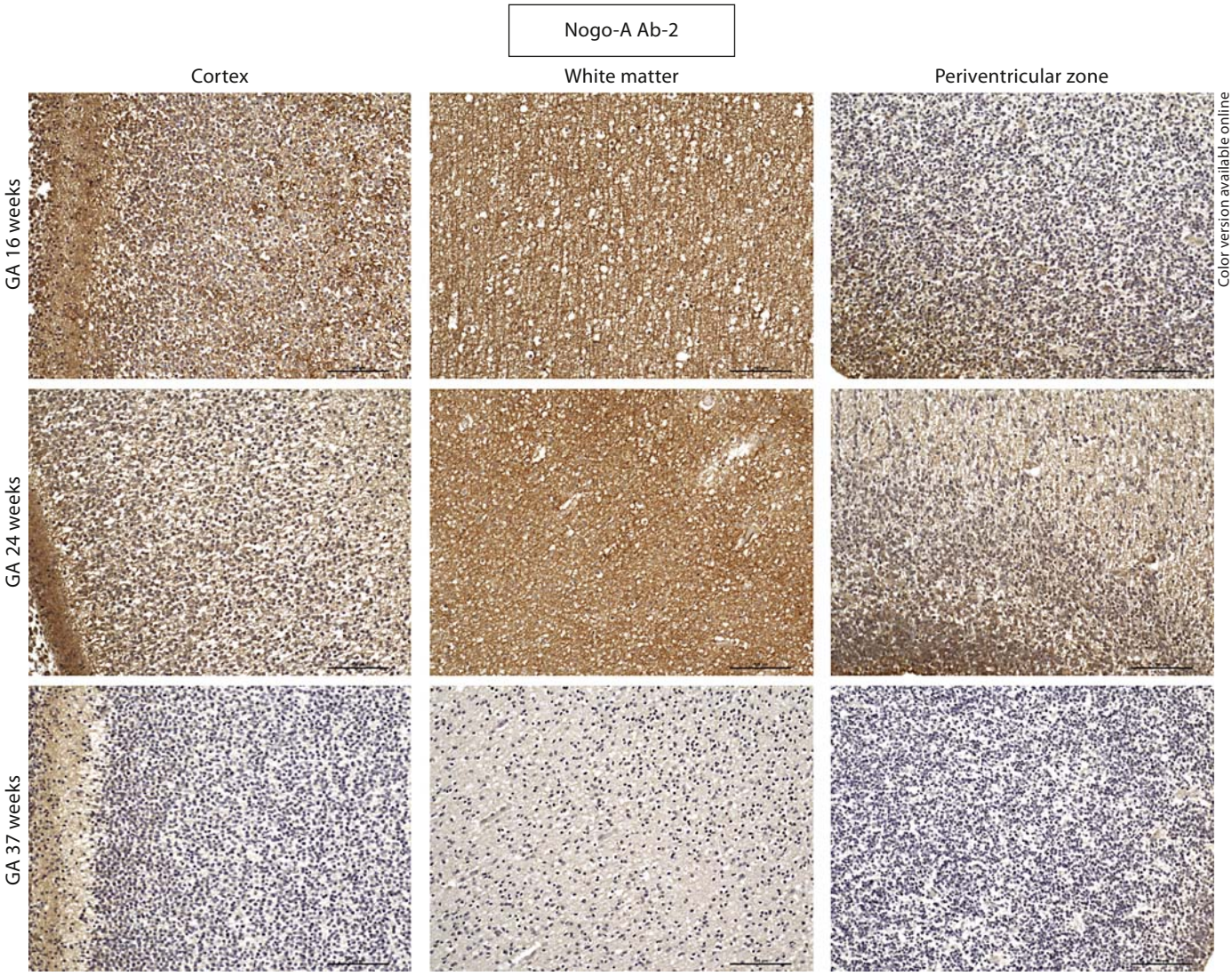

Fig. 4. Representative micrographs of Nogo-A immunopositive cells (stained with Ab-2) of three different age categories (GA 16, 24, 37 weeks) in the cortex, white matter and periventricular zone (magnifications indicated by scale bars, bar length corresponds to 100 μm). For colors, see online version.

There was a significant negative correlation between Nogo-A Ab-1-positive cells in the subplate and marginal zone and GA, as well as between Nogo-A Ab-2-positive cells in the marginal zone and GA (table 3). Thus, Nogo-A immunoreactivities are decreased with increasing GA in specific cortical areas.

For each antibody, the correlation between the various locations is shown in table 4. For Nogo-A Ab-1 immunoreactive cells, the following significant positive correlations were noted: (1) between subplate and subventricular as well as intermediate zones, (2) between the cortical plate and the ventricular zone, the intermediate zone and the subplate, and (3) between the marginal zone and ventricular, subventricular zone, subplate and cortical plate. For Nogo-A Ab-2, the positive cells in each region correlated positively with those in all other regions. Thus, in each zone, an increase of Nogo-A immunopositive cells resulted in an increase in the other zones.

Results were re-evaluated in a validation set using the well-established rabbit anti-Nogo-A ('Laura') antibody [18]. By using this antibody, all results found in our primary study set were reproducible (fig. 5, 6).

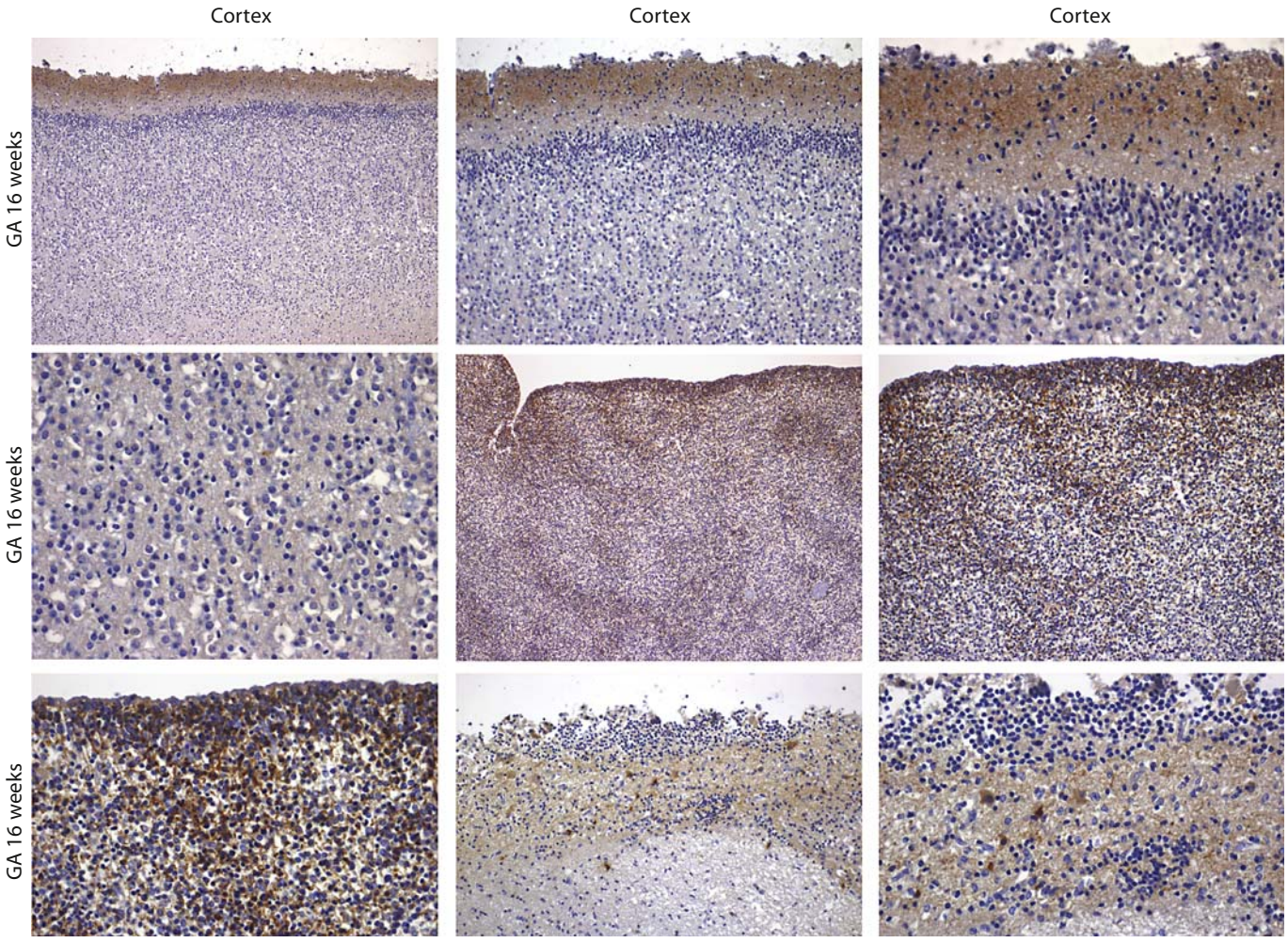

Fig. 5. Representative micrographs of Nogo-A ('Laura') immunopositive cells (stained with Ab-1) of GA 16 weeks in the cortex and adjacent white matter (magnifications ×20, ×40, ×60 from left to right). For colors, see online version.

Table 2. Differences in staining between both antibodies for the various regions studied

	Antibody 1		Antibody 2		p
	mean	SEM	mean	SEM	
Ependyma	3.00	0.00	2.46	0.24	*0.02*
Ventricular zone	2.00	0.21	1.07	0.30	*0.03*
Subventricular zone	1.40	0.16	0.71	0.22	*0.02*
Intermediate zone	1.77	0.13	1.33	0.21	0.07
Subplate	1.31	0.12	0.83	0.21	*0.01*
Cortical plate	1.50	0.16	1.13	0.21	0.08
Marginal zone	2.27	0.11	1.33	0.23	*0.00*

p values in italics are significant.

Table 3. Correlation coefficients (r) and p values between staining intensities and GA

	Antibody 1		Antibody 2	
	r	p value	r	p value
Ependyma	–	–	0.21	0.48
Ventricular zone	–0.13	0.67	0.07	0.82
Subventricular zone	–0.41	0.13	0.20	0.49
Intermediate zone	–0.15	0.42	0.15	0.45
Subplate	–0.39	*0.04*	–0.03	0.86
Cortical plate	–0.32	0.09	–0.03	0.86
Marginal zone	–0.41	*0.03*	–0.39	*0.04*

p values in italics are significant.

Table 4. Correlation coefficients (r) and p values between the various regions for each antibody (Ab) studied

	Ventricular zone		Subventricular zone		Intermediate zone		Subplate		Cortical plate		Marginal zone	
	r	p value	r	p value	r	p value	r	p value	r	p value	r	p value
Ab-1												
Ependyma												
Ventricular zone			0.43	0.12	0.39	0.17	0.52	0.06	0.64	*0.01*	0.57	*0.03*
Subventricular zone					0.22	0.44	0.61	*0.02*	0.58	*0.02*	0.59	*0.02*
Intermediate zone							0.48	*0.01*	0.56	*0.00*	0.32	0.09
Subplate									0.80	*0.00*	0.56	*0.00*
Cortical plate											0.66	*0.00*
Ab-2												
Ependyma	0.70	*0.01*	0.57	*0.04*	0.86	*0.00*	0.63	*0.02*	0.68	*0.01*	0.70	*0.01*
Ventricular zone			0.88	*0.00*	0.88	*0.00*	0.93	*0.00*	0.81	*0.00*	0.68	*0.01*
Subventricular zone					0.78	*0.00*	0.94	*0.00*	0.86	*0.00*	0.77	*0.00*
Intermediate zone							0.79	*0.00*	0.67	*0.00*	0.55	*0.00*
Subplate									0.79	*0.00*	0.78	*0.00*
Cortical plate											0.79	*0.00*

p values in italics are significant.

Discussion

The extraordinary rapid growth and plasticity of the nervous system and the major changes in cell and tissue properties occurring during development are due to different protein expression patterns. Nogo-A was first described in 2000 [3, 21, 22]. As an axonal regrowth inhibitor, Nogo-A plays an important role in regeneration and tissue development although its role in brain development remains unclear. Since the discovery of the Nogo protein as an individual myelin component capable of mediating the inhibition of axonal regeneration, the identity of axonal regrowth inhibitors, their physiological roles and their mechanisms of action have become partly clarified [23]. Several studies currently aim at blocking Nogo-A for therapeutic strategies in order to improve axonal regeneration in spinal cord injury. These targeted therapies have already been directed towards blocking interactions between Nogo and its receptor [24–28].

In the present study, Nogo-A was expressed in ependyma, ventricular zone, subventricular zone, intermediate zone, subplate, cortical plate, and marginal zone. The number of immunopositive cells decreased significantly with increasing GA in the subplate and marginal zone. Nogo-A expression was located in small cells with round nuclei resembling glial cells as well as neurons. The data we present here illustrate that expression of Nogo-A is important during early development. Ab-1 was targeted towards the Amino-Nogo epitope and showed a more complicated pattern of binding with GA than Ab-2, which was targeted to an epitope adjacent to the Nogo-66 region of the protein.

As it is the amino terminus that contains the sequence that differs between various reticulon genes (reviewed by Yang and Strittmatter [1]), the data for Ab-1 are more likely to represent functions unique to Nogo-A (or its isoforms, including Nogo-B) as compared to other proteins of the reticulon family. Yet in terms of the isoforms of Nogo, it is antibody Ab-1 that is more specific for Nogo-A. For Ab-1, correlations were observed only between some regions with GA, whereas Ab-2 showed consistent correlations between regions with GA, i.e. the intensities for Nogo-A expression decreased significantly with increasing age. The divergent outcome for Ab-1 and Ab-2 either reflects different regional patterns of expression of Nogo-A versus Nogo-B, or differential dimerization of Nogo-A depending on the GA, as the coiled-coil formation which underlies dimerization at the N-terminus would be expected to disrupt the binding of Ab-1 to the Amino-Nogo epitope, whereas the binding of Ab-2 to the protein would be unaffected by dimerization. While antibody Ab-1 predominantly recognizes a band at approximately 50 kDa corresponding to the molecular weight of Nogo-B (fig. 2) as well as a band thought to correspond to

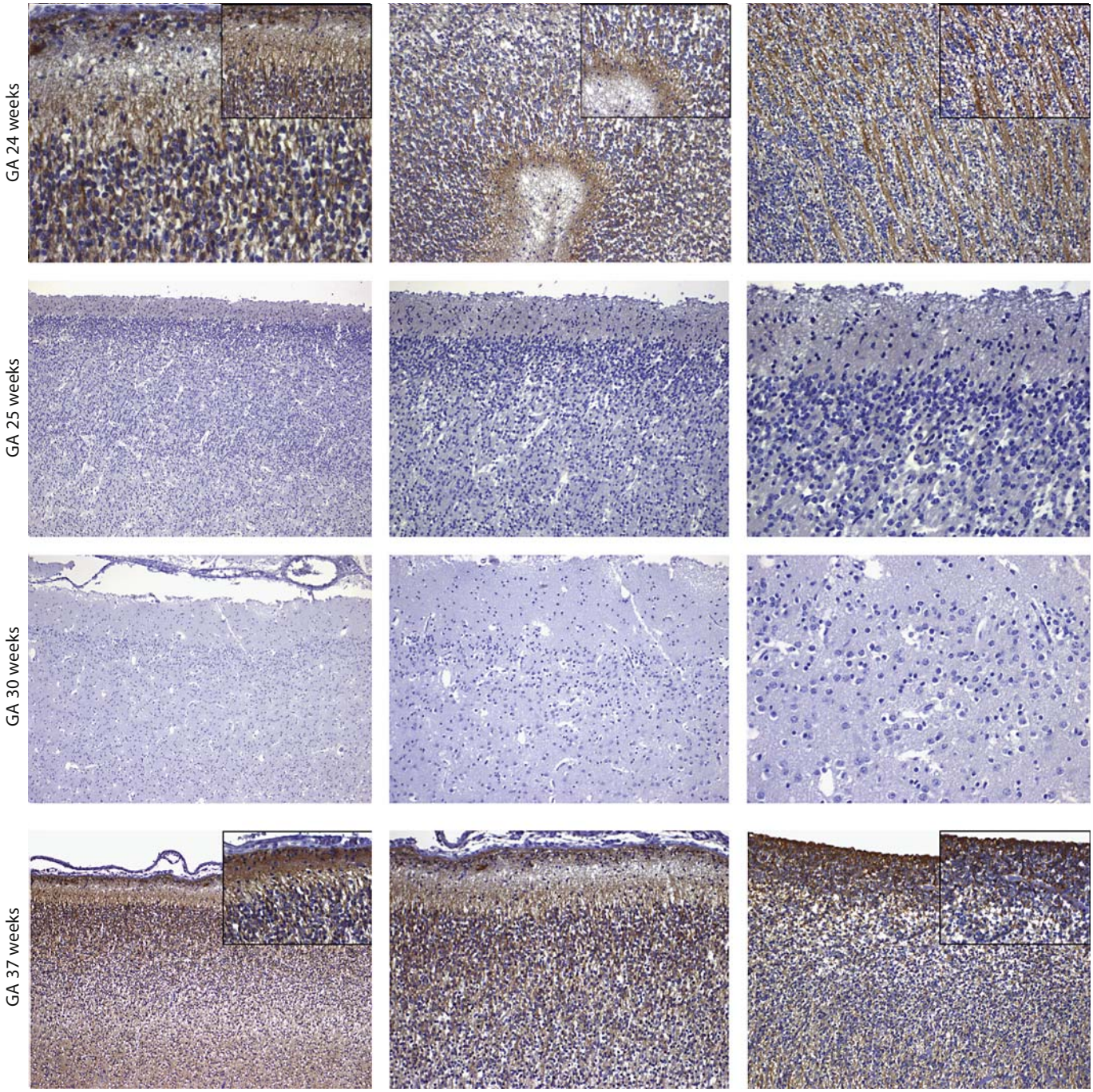

Fig. 6. Representative micrographs of Nogo-A ('Laura') immunopositive cells (stained with Ab-1) of four different age categories (GA 24, 25, 30, 37 weeks) in the cortex and adjacent white matter (magnifications ×20, ×40, ×60 from left to right; insets with higher magnification). For colors, see online version.

the monomer of Nogo-A at approximately 180 kDa, antibody Ab-2 is recognizing the monomer plus a band at 260 kDa, the correct size for a dimer of Nogo-A. This dimer is predicted to occur through interaction of the coiled-coil regions in the N-terminus. Because Ab-2 is directed towards a region distal from the coiled-coil regions, its recognition of the dimer is not impaired. In addition, antibody Ab-2 seems to be more specific than antibody Ab-1 as it does not detect the band migrating at the location of Nogo-B (approx. 50 kDa).

Correlation between regions showed that for Ab-2 the staining intensity in one compartment paralleled that in the other compartments. For Ab-1, however, the staining intensity did not correlate between the ventricular zone and the subventricular or intermediate zone as well as between the subventricular zone and the intermediate zone.

It has become increasingly apparent that Nogo-A probably has a variety of roles. Knocking out the gene for Nogo or the NgR does not result in a severe phenotype under physiological conditions and changes of the regenerative capacity of injured CNS. Three independent groups reported different and at least partly contradictory results [29, 30].

Among the many clues that *Nogo* transcripts might have other roles is the growing number of possible interaction partners besides the NgR [30, 31]. Like other members of the reticulon family, Nogo is an endoplasmic reticulum-enriched protein, and interactions with other endoplasmic reticulum, mitochondrial and cytoplasmic proteins may be important for various cellular physiological processes. The fact that Nogo-deficient mice apparently exhibit a normal physiological phenotype could be related to the compensatory roles that other members of the reticulon family might perform in normal physiology [31, 32].

As previously shown, Nogo-A expression in adult neurons does not appear to be influenced by the local presence of inflammatory cytokines or neurotrophic factors [33]. At a cellular level, Nogo-A and NgR are expressed in a pattern consistent with their role in axonal-glial interactions and limitation of axonal sprouting in the adult CNS [34]. NgR is expressed in mature neurons, and Nogo-A in the adaxonal myelin sheath and in the outermost myelin membranes [11]. The expression of Nogo-A is not significantly altered after CNS injury, unlike other myelin molecules. Its role in neurite growth inhibition under physiological conditions seems to be restricted to the developing nervous system, and after that to tonic inhibition of adult neuronal growth [11, 35, 36].

Nogo-A is known to be highly expressed in oligodendrocytes of higher vertebrates, where it localizes mainly to the outer and innermost axonal myelin sheath and to synaptic sites. During development, oligodendrocytes show an expression pattern which directly correlates with myelination. In the cerebellum, *Nogo*-A mRNA appears in oligodendrocytes in deep cerebellar areas at P5 and later on, at P9. Nogo-A-expressing neurons are detected at the distal ends of the folia in the white matter [11, 37, 38]. Although developing neurons express Nogo-A, this protein is not expressed in most adult neurons. Olfactory receptor neurons as well as cerebellar granular cells show high levels of mRNA during development, whereas in these cells Nogo-A is downregulated after maturation. During development, neurons and glial cells are the major source of Nogo-A. Nogo-A seems to be regulated by a gradient of positioning and maturation of the cerebral cortex [17]. As its expression is postmitotic, it is first seen in the preplate (E11–E12) before the division of this structure into the subplate and marginal zone, followed by the expression of postmitotic cells in the emerging cortical plate. In lower vertebrates, which are known to have a high regenerative competence, Nogo-A is not found in the CNS. This stands in contrast to mammals.

In mice, tangentially and radially migrating neurons display different expression patterns. The genetic ablation of *Nogo* leads to a delay in the tangential migration of GABAergic interneurons. It was reported that neuronal NgR expression in the neocortex does not start until late prenatal and early postnatal stages [13, 36]. The latter finding suggests no functional mediation by NgR at this developmental stage, and points to the interaction of Nogo with other effectors. Interestingly, the immunohistochemical expression of Nogo-A in ependymal cells has not yet been documented in detail.

Recently, an analysis of Nogo-A mRNA and protein expression pattern in the embryonic mouse forebrain was performed [17]. During embryonic development, Nogo-A was expressed by radial glia throughout corticogenesis. Neuronal Nogo-A protein was expressed in postmigratory cortical neurons, predominantly localized to the growing axon. Tangentially migrating GABAergic neurons from the ganglionic eminence expressed Nogo-A, targeting the protein to their leading processes.

Nogo-mutant mice showed no significant changes in axonal tracts, although absence of *Nogo* resulted in an altered migratory behavior of early GABAergic neurons during corticogenesis. Moreover, an increase in axon branching and early polarization was described in vitro in *Nogo*-deficient murine neurons [39, 40] and preceded

NgR expression [13]. Nogo-A expression was observed to be highly expressed in some murine telencephalic axonal tracts during early embryonic development [12, 13, 41], once again indicating that *Nogo*-A functions independently from NgR at this stage, and likely participates in axonal tract formation or neurite growth. The expression pattern at perinatal stages of animal CNS development has been reported in various studies [12, 13, 34, 36, 41–43].

Nogo mRNA expression was first observed as early as E12.5. In the hippocampus, predominantly in the hippocampal preplate, Nogo-A was already seen at E12.5. It became enriched in the CA1–CA3 regions by E14.5. Moreover, Nogo-A antibody highlighted cortical afferents and efferents such as the corticothalamic and thalamocortical tracts, the hippocampal fimbria, the corpus callosum, the anterior commissure, and the lateral olfactory tract [17]. Nogo-A-positive cells were radially oriented including the cortical width at E12.5. Double immunostaining of Nogo-A polyclonal antibody and Nestin in the cortex of E12.5 and E18.5 mice could be demonstrated to precisely colocalize in radial glia at E12.5 and partially at E18.5, when Nogo-A was still seen at glial end feet. At E12.5, Nogo-A was detected in pioneering neurons in the preplate contrasting with the nonneuronal Nogo-A radial glial pattern.

Other authors have proposed that Nogo probably participates in the migration process of early GABAergic neurons to the cortex and delays the migration of E12.5-generated interneurons toward the neocortex. Cortical GABAergic interneurons generate from the ganglionic eminence and migrate through the intermediate and subventricular zone before integrating into the cortical plate [44–49]. Between E13.5 and E16.5, a band of tangential processes immunoreactive for Nogo-A was seen in the lower intermediate and subventricular zone.

At E14.5, Nogo-A staining was found throughout the entire rostrocaudal extent of the telencephalon. Nogo-A labeling followed a rostrocaudal gradient in the cerebral cortex. Nogo-A protein and mRNA were detected in the pyramidal cell layer at E14.5 [14]. During later development, at E15.5, Nogo-A immunoreactivity was prominently shown in cortical axonal tracts, in the medial telencephalon and the anterior commissure. At E15.5, Nogo-A protein was absent from the perikaryon of neurons located in the lower cortical plate but present in corticofugal axons. At E18.5, Nogo-A was enriched in the corticocortical connections of the corpus callosum, the anterior commissure, and the lateral olfactory tract [17]. Nogo mRNA was also detectable in the cerebral cortex and subcortical regions like the striatum. In the developing cortex, *Nogo* mRNA was found in the lower portion of the cortical plate (layers VI–V) and the subplate layer VIb [17]. Surprisingly, Nogo-A was expressed by radial glial cells from both the ventral and the dorsal telencephalon. *Nogo*-deficient mice displayed a 25% reduction in the number of E12.5-generated interneurons compared with control littermates but not in the number of E15-generated interneurons [17].

Studies by Metin and Godement [50] demonstrated that early generated interneurons (E11–E13) in the medial ganglionic eminence use the corticofugal tract to reach the dorsal pallidum by migrating in close contact with corticofugal fibers. In another study, a specific decrease in the number of early generated interneurons (E12.5 cohort) that populate the somatosensory cortex was found, possibly indicative of the participation of *Nogo* in this process [17]. These data suggest that Nogo-A may also regulate tangential migration by acting as an adhesion molecule in the corticofugal tract although Nogo-A's main function is anti-adhesive.

Nogo-A labeling in growth cones has been shown to be restricted to the central region and matched microtubule distribution. Cell culture experiments indicated that Nogo proteins are required for appropriate branching pattern in cultured neurons and that the absence of these proteins leads to early neuronal polarization [17].

Our data from the examination of human brain tissues confirm the reported murine data in a sense that significant changes in the expression pattern of Nogo-A during early development can be described. As the results for Ab-1 and Ab-2 diverge, it is likely that Ab-2 is more specific for Nogo-A, whereas Ab-1 recognizes both Nogo-A and Nogo-B. We conclude that Nogo-A plays an important role in cortical development at various GAs and in different brain locations. Dimerization of Nogo-A was found to occur only in white matter at one developmental time point. Whether this finding holds true across developmental stages awaits future studies.

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